5359
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Evaluation of strains of *Geotrichum candidum* for lipase production and fatty acid specificity

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Summary. Three strains of *Geotrichum candidum* (ATCC 34614, NRRL Y-552 and NRRL Y-553) were examined for lipase production and activity. Variables including medium, pH, temperature, agitation rate and incubation time were examined to define the optimal culture conditions. Growth on oil in complex medium at 30° C, 300 rpm, and pH 7 produced maximal lipase activity. Fatty acid specificity of these strains and of two crude *G. candidum* enzyme preparations (lipase 26557 RP, Rhône Poulenc and lipase GC-4, Amano) was measured using equimolar mixtures of methyl or butyl esters of palmitic and oleic acids. The lipase from NRRL Y-553 and lipase 26557 RP displayed preferential specificity for hydrolyzing oleic acid esters, while the lipases from ATCC 34614, NRRL Y-552 and lipase GC-4 failed to discriminate between palmitic and oleic acids.

Introduction

Lipase (EC 3.1.1.3) from *Geotrichum candidum* has a unique specificity for unsaturated fatty acids with a double bond(s) at position *cis*-9 or *cis, cis*-9, 12; esters of these fatty acids are hydrolyzed much more rapidly than most others (Jensen 1974; Macrae 1983). The original strain showing this specificity (Alford and Pierce 1961), further characterized by Jensen (1974), is no longer available (personal communication, Drs. John A. Alford, Arthur R. Colmer, Robert G. Jensen and James L. Smith). Lipase activity from various available strains of *G. candidum* shows interesting variations. In ATCC 34614, lipase activity must be induced (Iwai et al. 1973; Tsujisaka et al. 1973), while in other strains it is constitutive (Alford and Smith 1965; Chander and Klostermeyer 1983). The degree of specificity for unsaturated fatty acids also varies (Jensen and Pitas 1976; Okumura et al. 1976). Lipase from ATCC 34614 has been purified and its low resolution X-ray structure determined (Hata et al. 1979). We have chosen three strains and two enzyme preparations of *G. candidum* to study lipase structure and to evaluate lipase use for interconverting or hydrolyzing fats and oils. Strains showing the reported specificity for unsaturated fatty acids (Jensen 1974; Macrae 1983) could be of industrial interest for fat splitting and production of specialty chemicals from fats and oils.

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Materials and methods

G. candidum lipase 26557 RP (lipase RP), 0.47 units/mg, was a kind gift from Rhône Poulenc, France and *G. candidum* lipase GC-4 from Amano International Enzyme Company, Troy, Virginia. Olive oil was purified according to the procedure of Linfield et al. (1984).

Microorganisms. G. candidum ATCC 34614 was obtained from the American Type Culture Collection, Rockville, Maryland. *G. candidum* NRRL Y-552 and NRRL Y-553 were obtained from the Agricultural Research Service Culture Collection, Peoria, Ill. The cultures were maintained at 4° C on malt extract agar slants: 2% each malt extract (Difco Laboratories, Detroit), glucose and agar, 0.1% peptone (Difco) with monthly transfer.

Media. Two basal media were tested for support of lipase production. The first was a modification of the medium of Tsujisaka et al. (1973), with glucose omitted. This medium contained 5% peptone, 0.1% $NaNO_3$ and 0.1% $MgSO_4$ adjusted to the desired pH before sterilization. Sterilization did not cause a significant pH change. Sterile soybean oil (Sigma Chemical Company, St. Louis), olive oil, glycerol, sucrose or glucose was added as a carbon source. The completely defined medium of Smith and Alford (1966) was also tested briefly.

Lipase production. Starter cultures were obtained by transferring a loopful of cells from an agar slant culture to 65 ml of sterile medium in a 250 ml Erlenmeyer flask and incubating 24 h at 30° C and 300 rpm on a rotary shaker. One milliliter of starter culture was transferred into 65 ml fresh medium in a 250 ml Erlenmeyer flask for each experiment. Lipase activity was first detectable in the late logarithmic phase of growth.

Lipase assay. Lipase activity was measured by a modification of the assay of Parry et al. (1966) using as substrate a 10% olive oil-gum arabic solution emulsified by sonication for 2 min at 25 watts output according to Linfield et al. (1985). One milliliter of cell-free fermentation broth prepared by centrifugation was added to 5 ml of emulsion and incubated at room temperature for 1 h with rapid stirring. Ethanol was added to stop the reaction, and the free fatty acids produced were quantified by titration to pH 9.5 with 0.0200 or 0.100 N NaOH using a Radiometer titration system. Blanks with 1 ml of fermentation broth were employed with each experiment. Blanks run with sterile or actual, unincubated broth were the same within experimental error. All samples were run in duplicate. The standard deviation of the assay is ± 10% or less. A unit of lipase activity is defined as the release of one micromole of free fatty acid (FFA)/min at room temperature.

Acetone powder. Lipase was precipitated from the fermentation broth using the acetone powder method of Morton (1955). Recovery of activity averaged 67% ± 21%.

Substrate specificity. To determine the specificity of the lipase preparations, substrate esters of oleic ($C_{18:1}$) and palmitic (C_{16}) acids were hydrolyzed simultaneously in the presence of enzyme. Reaction mixtures consisted of: 500 μmole of a mixture of the methyl or butyl esters; 4 ml of pH 7.0 buffer containing 0.05 M Tris, 0.01 M $CaCl_2$; 25 drops of 10% gum arabic (Sigma), and 1—2 units of lipase. The gum arabic served to emulsify the substrate esters, a homogenous oil phase, in the buffer solution. Reaction mixtures were incubated at 30° C with rapid stirring and titrated to determine free fatty acid production and percent conversion. The relative amounts of unreacted esters were determined by gas chromatography at 280° C on a fused silicia SPB-1 capillary column (0.25 mm I.D. × 30 m). Assuming irreversibility and lack of product inhibition, these values allowed calculation of the ratio of the specificity constants, V_{max}/K_m, for two competing substrates A and B based on the following expressions (Chen et al. 1982).

$$\frac{(V_{max}/K_m)_A}{(V_{max}/K_m)_B} = \frac{\ln(A/A_0)}{\ln(B/B_0)} = \frac{\ln[(1-c)(1-SE)]}{\ln[(1-c)(1+SE)]}$$

Here V_{max} denotes the maximal velocity, K_m is the Michaelis-Menten constant, c is the conversion in mole fractions, and SE is the excess of one substrate over total remaining substrate at conversion c. Note that ratios calculated at low percent conversions have larger relative error. Originally derived for measuring the relative rates of reaction of enantiomers during resolution of racemates, the method should be equally useful for characterizing relative specificity for pairs of compounds undergoing competitive enzymatic catalysis. Recently, similar competitive esterification reactions in organic solution were successfully used to study the mechanism of crude lipases from *Candida rugosa* and *Mucor meihei* and to predict kinetic separations and resolutions by the lipases (Deleuze et al. 1987).

The selectivity of lipase RP was further studied by hydrolyzing olive oil. Lipase (4.7 units), olive oil (1.52 g), buffer (10 ml of 0.05 M Tris, 0.01 M $CaCl_2$, pH 8.0) and 10% gum arabic (25 drops) were reacted and analyzed as above.

Results

Effect of medium composition

Preliminary results showed that growth on completely defined medium resulted in one third the lipase activity of growth on complex medium. Use of the defined medium was therefore abandoned; all results reported here were obtained using complex medium. No lipase was produced by *G. candidum* grown on glucose or sucrose. Lipase production on glucose and oil was less than that on oil alone (Table 1). Equivalent results were obtained using soybean oil or olive oil.

Table 1 also shows lipase activity produced with glycerol as the carbon source. Maximal lipase production is lower in glycerol than in oil for ATCC 34614 and NRRL Y-552, but similar or higher for NRRL Y-553. Maximal lipase activity is produced with 0.057 N (0.5% v/v) glycerol by ATCC 34614, and with 0.230 N glycerol by NRRL Y-552 and Y-553 in 48 h.

Effect of pH, temperature and agitation rate

Table 2 shows the effect of environmental variables on lipase production using 1% (v/v; 0.048 N) soybean oil. The medium was adjusted to pH 5.4, 6.4, 7.0 or 7.4 (30° C, 300 rpm). Growth at pH 7.0 resulted in maximal lipase activitiy at 24 h compared to later and generally lower peak activity under more acidic or basic conditions.

Table 1. Effect of carbon source on lipase production at 30° C, pH 7.0, 300 rpm

Carbon Source[a]	Concentration (N)	Time (hours)	Activity (unit/ml)		
			34614	Y-552	Y-553
Soybean oil	0.057	24	5.2	7.2	2.4
		48	4.0	6.2	3.4
		72	4.8	7.3	1.4
Soybean oil + Glucose	0.057 / 0.057	24	0.0	0.7	0.1
		48	2.3	2.1	2.2
		72	4.0	2.5	0.0
Glycerol	0.057	48	2.8	1.6	2.5
	0.115	48	2.5	2.1	3.6
	0.230	48	2.2	2.4	5.4
	0.344	48	1.9	1.7	2.1

[a] All three strains were also grown on 0.057 N glucose. No lipase activity was found at 24, 48 or 72 h

Table 2. Effect of pH, temperature and agitation on lipase production with 1% soybean oil

Variable		Time (hours)	Activity (unit/ml)		
			34614	Y-552	Y-553
pH	5.4	24	2.9	2.7	2.7
		48	2.7	3.0	2.6
		72	2.3	4.2	2.5
		96	2.6	3.7	1.0
	6.4	24	2.4	3.9	2.3
		48	3.4	4.1	2.8
		72	4.0	4.9	2.5
		96	2.2	3.5	0.6
	7.0	24	3.7	5.5	5.5
		48	3.0	3.8	3.0
		72	2.8	3.8	1.6
		96	1.4	3.6	0.5
	7.4	24	2.4	3.8	3.1
		48	2.9	3,7	3.2
		72	2.7	3.6	2.1
		96	2.2	2.6	0.2
Temperature (°C)	20	24	3.4	4.1	2.8
		48	3.3	3.0	3.1
		72	3.2	3.4	2.4
		96	3.6	3.9	2.1
	30	24	3.7	5.5	5.5
		48	3.0	3.8	2.8
		72	2.8	3.8	1.6
		96	1.4	3.6	0.5
	40	24	0.1	0.0	0.0
		48	0.2	0.2	0.2
Rpm	0	24	0.6	0.6	0.6
		48	1.6	1.4	1.4
	100	24	1.6	1.4	1.1
		48	1.1	0.5	0.5
	200	24	2.0	3.4	2.2
		48	1.5	3.5	2.0
		72	1.5	3.6	1.4
		96	2.1	3.0	1.6
	300	24	3.7	5.5	5.5
		48	3.0	3.8	2.8
		72	2.8	3.8	1.6
		96	1.4	3.6	0.5

Temperature was varied from 20–40°C (pH 7.0, 300 rpm). No or very little lipase was produced at 40°C. More activity was present at 30° than at 20°, but the activity was maintained longer in the culture broth at 20°C. Similar results were found for stationary growth.

The agitation rate was varied from 0 to 300 rpm (30°C, pH 7.0). Maximal lipase activity was found at 24 h and 300 rpm, and decreased at lower rpm as expected for an aerobic organism.

Specificity

Table 3 shows the relative specificity constants for hydrolysis of oleic and palmitic esters, $(V_{max}/K_m)_{18:1}/(V_{max}/K_m)_{16}$, for lipases of the three strains, lipase RP and lipase GC-4. ATCC 34614, NRRL Y-552 and lipase GC-4 were moderately specific for oleates at best (ratio close to 1), while NRRL Y-553 and lipase RP showed good specificity (ratio much greater than 1) with both methyl and n-butyl esters. Further experimentation was confined to NRRL Y-553 and/or lipase RP due to this higher specificity.

Table 3. Relative specificity constants of oleic and palmitic esters

Lipase	$\frac{(V_{max}/K_m)_{18:1}}{(V_{max}/K_m)_{16}}$	
	Methyl	n-Butyl
34614	1.0	4.1
Y-552	1.5	1.0
Y-553	37	>50[a]
lipase RP	>50[a]	>50[a]
lipase GC-4	4.1	ND[b]

[a] Low conversion (<20%) causes a higher experimental error

[b] Not determined

Figure 1 shows a loss of specificity during hydrolysis of methyl esters for lipase RP and the acetone powder of the cell-free culture broth of NRRL Y-553. This loss of specificity during reaction appeared to correlate with percent conversion rather than time of reaction. In addition, we noted that the acetone powder derived from NRRL Y-553 showed lower specificity (12:1 for 27% conversion of methyl esters) than did the culture broth itself (37:1 for 14% conversion).

Figure 2 shows the major free fatty acids produced during the hydrolysis of olive oil by lipase

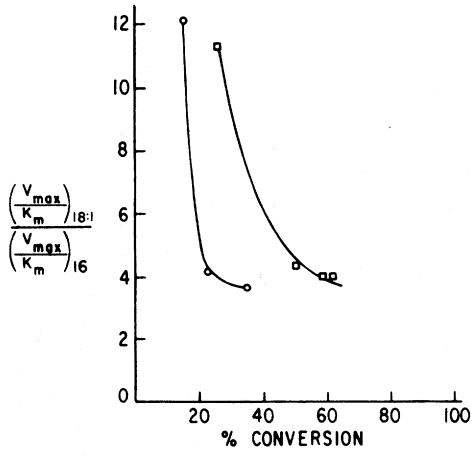

Fig. 1. Loss of selectivity during competitive hydrolysis of methyl esters of oleic and palmitic acids. *G. candidum* lipase: o, NRRL Y-553; □ lipase RP

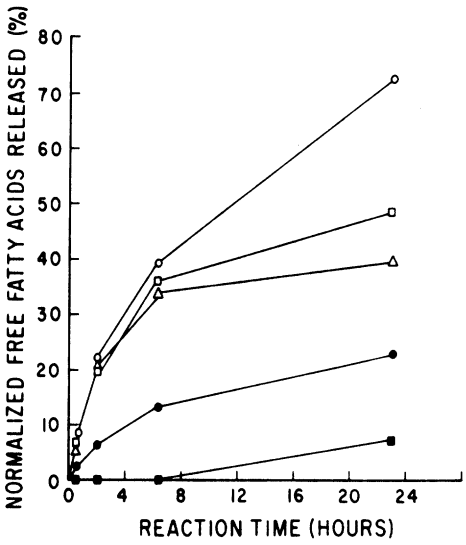

Fig. 2. Hydrolysis of olive oil by *G. candidum* lipase RP. The major free fatty acids released are shown as a function of time, normalized to their composition in unreacted olive oil. ○, palmitoleic ($C_{16:1}$); □, oleic ($C_{18:1}$); △, linoleic ($C_{18:2}$); ●, palmitic ($C_{16:0}$); ■, stearic ($C_{18:0}$)

RP. We chose to add the unsaturates versus saturates to calculate an apparent specificity constant ratio, $(V_{max}/K_m)_u/(V_{max}/K_m)_s$, of greater than 4:1 for 6–7% conversion at 0.5 h. The specificity declined thereafter to approximately 2.4:1 after 30.9 and 42.8% conversion (6.3 and 23 h respectively).

Preliminary isoelectric focusing experiments showed that all five enzyme preparations have multiple lipase and esterase activities (data not shown). We are attempting to purify *G. candidum* lipase and determine its specificity.

Discussion

For all three strains of *G. candidum* studied here, lipase production appears to be stimulated by the presence of oil or glycerol. This agrees with the results reported for ATCC 34614 (Iwai et al. 1973; Tsujisaka et al. 1973). Glucose and sucrose appear to inhibit lipase production.

Lipase production for all three strains grown on oil is highest and most rapid at 30° C, 300 rpm, and neutral pH. Lipase activity of culture filtrates decreased with time. This phenomenon has been noted previously (Hassanien et al. 1985; Iwai and Tsujisaka 1984) and the decrease has been attributed to the presence of proteases. We plan to study the correlation of cell growth, lipase and protease production to further optimize lipase production.

The specificity of the *G. candidum* lipases apparently varies greatly with strain. Jensen and Pitas (1976) report small variations between the strains used by Alford and Pierce (1961) and by Franzke et al. (1973). Okumura et al. (1976) report that lipase from strain ATCC 34614 releases both oleic and palmitic acids, in agreement with our results.

The ratio of specificity constants is not expected to alter during the course of the reaction. This ratio is an intrinsic property of the substrates and enzyme, and is expected to remain constant (Chen et al. 1982), in contrast to the declining selectivity shown in Fig. 1. The decline could be due to the presence of multiple enzymes varying in stability or to product inhibition.

Industrial utility hinges on obtaining a "usefully" high rate ratio and an enzymatic preparation that retains specificity during operation. Operationally, we feel that relative rate ratios in the range of 10–15:1 would be industrially useful for separation of unsaturated fatty acids. Clearly the two more selective preparations may meet that criterion. Studies are currently being conducted to determine the basis of lipase specificity and to understand the loss of that specificity during reactions.

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